Establishment of HIV-1 model cell line GHOST(3) with stable DRiP78 and NHERF1 knockdown

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ABSTRACT

Chemokine receptors CXCR4 and CCR5 are indispensable co-receptors for HIV-1 entry into host cells. In our previous study, we identified that dopamine receptor-interacting protein 78 (DRiP78) and Na^+-H^+ exchanger regulatory factor 1 (NHERF1) are the CXCR4 and CCR5 homo- or hetero-dimer-interacting proteins. DRiP78 and NHERF1 are able to influence the co-receptor internalization and intracellular trafficking. Over-expression of NHERF1 affects the ligands or HIV-1 gp120-induced CCR5 internalization and HIV-1 production. It is reasonable to speculate that DRiP78 and NHERF1, as well as the signaling pathways involved in viral replication, would probably affect HIV-1 replication through regulating the co-receptors. In this present study, we designed two short hairpin RNAs (shRNAs) targeting the DRiP78 and NHERF1, respectively, and constructed the pLenti6/BLOCK-iT-DEST lentiviral plasmids expressing DRiP78 or NHERF1 shRNA. The packaged lentiviruses were used to transduce the widely-applied HIV-1 model cell line GHOST(3). Then, cells with stable knockdown were established through selecting transduced cells with Blasticidin. This study, for the first time, reported the establishment of the GHOST(3) with DRiP78 and NHERF1 knockdown, which is the first stable cell line with HIV-1 co-receptor-interacting molecular defects.

Keywords: HIV-1; DRiP78; NHERF1; shRNA; GHOST(3) cells

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is the etiological agent of human acquired immunodeficiency syndrome (AIDS) that severely threats human health worldwide during the past three decades. The chemokine receptors CXCR4 and CCR5 are co-receptors for HIV-1 entry into host $CD4^+$ cells. Their ligands can strongly inhibit the replication of HIV-1 (Deng et al, 1996; Feng et al, 1996). Previous studies showed that after infection, CXCR4- and CCR5-tropic HIV-1 could activate different signaling pathways and thereafter result in different gene expression profiles (Cicala et al, 2006), suggesting that these two receptors can mediate different internalization and intracellular transport pathways to complete the infection and replication of the virus into host cells. Dopamine receptor-interacting protein 78 (DRiP78), also known as DnaJC14, Jiv or HDJ3, is a member of the heat shock proteins of the Hsp40 family (Kelley, 1998). Proteins of this family contain a J model domain composed of 70 amino acids, which plays an important role in raising the Hsp70 family membership and stimulating ATP hydrolysis during chaperone processing. DRiP78 is a molecular chaperone binds to endoplasmic reticulum and is involved in the regulations of a variety of Guanosine-binding protein coupled receptors (GPCR), including D1 dopamine receptor, M2 muscarinic receptor, AT1 angiotensin II receptor, adenosine receptor and the β2 adrenergic receptor of cell membrane transport (Bermak et al, 2001; Dupré et al, 2007; Leclerc et al, 2002; Málaga-Diéguez et al, 2010). Meanwhile, DRiP78 is also involved in the regulations of $G_{\beta\gamma}$ subunit of G protein assembly (Dupré et al, 2007).

Na^+-H^+ exchanger regulatory factor 1 (NHERF1) is an adaptor protein binds to a variety of GPCR. NHERF1, also called Ezrin-Radixin-Moesin binding phosphate phosphoprotein 50 (EBP50), is consisted with three functional domains,

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including two N-terminal tandem PDZ domains and one C-end Ezrin-Radixin-Moesin (ERM) domain (Hung & Sheng, 2002). NHERF1 is an important factor of protein kinase in inhibiting Na^+/H^+ exchange isoform 3 (NHE-3) and was initially identified in 1987 (Weinman et al, 1995). Studies showed that NHERF1 is involved in lots of events related with GPCR, ion channels, as well as transporter recycling and sorting (Cao et al, 1999; Lazar et al, 2004; Li et al, 2002; Wang et al, 2007).

Our previous studies showed that DRiP78 and NHERF1 were interacting proteins of HIV-1's co-receptors, CXCR4 and CCR5, but their recognition specificities were different (Hammad et al, 2010; Kuang et al, 2012a). DRiP78 recognizes and binds to the homologous dimers of CXCR4 and CCR5, but not the heterologous dimers of CXCR4-CCR5, whereas, NHERF1 binds to the homologous dimers of CCR5, but not the homologous dimers of CXCR4 nor heterologous dimers of CXCR4/CCR5.

DRiP78 promotes the formation of homologous dimers complex and the signal assembly of G protein subunit and CCR5, but cannot affect the assembly of heterologous dimers (Kuang et al, 2012a). DRiP78 regulates the signal complex of co-receptors CXCR4/CCR5, specifically, and influences the migration of receptor-mediated downstream biological function entry into immune cells (Kuang et al, 2012a). The molecular chaperone, DRiP78, may represent a novel class of target, which regulates the expression levels of receptors in the cytoplasm, and finally affects their binding on the cell surface of chemokine and related viruses, such as HIV-1. However, the effects of DRiP78 on HIV-1 entry and replication and the underlying mechanisms are still unclear. Therefore, the establishment of HIV-1 model cell line GHOST(3) with stable silencing of DRiP78 and NHERF1 is critical in understanding the roles of DRiP78 and NHERF1 play in the entry and replication of HIV-1 and the mechanisms involved.

MATERIALS AND METHODS

shRNA design and lentiviral plasmid construction

The nucleic acid sequences for RNA interference with human DRiP78 and NHERF1 were as follow: human DRiP78: 5′-CCG AGG AAC UAU GUC AAC UUG GAC A-3′ and human NHERF1: 5′-CAG AAG GAG AAC AGT CGT GAA-3′, respectively. The shRNA oligos of interest were synthesized by Invitrogen, China (Table 1).

The construction of shRNA-expressing lentiviral vector was outlined in Figure 1. The synthesized complementary oligo DNA was annealed and ligated with linear pENTR/U6-EGFP plasmid. The ligated product was transformed into DH5α competent cells and plated on LB plates containing 50 μg/mL Kanamycin, and then incubated inverted at 37 °C overnight. Three clonies were picked up from each plates and shaked at 37 °C overnight. The successful plasmids were named pENTR/U6-EGFP-shDRiP78 and pENTR/U6-EGFP-shNHERF1, respectively. Finally the plasmids were verified by sequencing.

Lentiviral interference vectors were constructed by LR clonase II recombining plasmid with the target vector pLenti6/BLOCK-iT-DEST, and ligated the target gene with the destination vector plasmid. These two recombinant plasmids were named pLenti6/BLOCK-iT-DEST-shDRiP78 and pLenti6/BLOCK-iT-DEST-shNHERF1, respectively. They were transformed into Stbl3 competent cells and plated on LB plates containing 100 μg/mL of Ampicillin. The plates were screened and monoclonies were picked up and were amplified in LB liquid medium containing 100 μg/mL Ampicillin. The plasmid was extracted using Axygen plasmid DNA kit and verified by direct sequencing.

Lentiviral package and preparation

The HEK293T cells on logarithmic phase were counted at the density of 6×10^6 cells/10 cm culture dish, and then were incubated at 37 °C, 5% CO_2, overnight. Before transfection, culture medium was replaced with 5 mL Opti-MEM medium (Invitrogen). Packaging mix (9 μg, Invitrogen) and lentiviral expression plasmid (3 μg) were added into 1.5 mL Opti-MEM and were mixed gently. Lipofectamine 2000 (36 μL, Invitrogen) was added into 1.5 mL Opti-MEM and was mixed gently, and then incubated at room temperature for 5 min. The diluted plasmid solution and diluted Lipofectamine 2000 were mix gently and were incubated at room temperature for 20 min. Plasmid-liposome complex (3 mL) was added carefully into the cells and was mixed gently, then incubated 6 hour in a 37 °C, 5% CO_2 incubator. Then, the medium was replaced with DMEM complete medium containing 10% fetal bovine serum (FBS). Fourty-eight hour later, the cell culture supernatants were collected, and were centrifuged at 3 000 g for 10 min to remove cells and debris, and then were filtered with a 0.45 μm filter. The clear viral supernatant was concentrated by ultracentrifugation at 50 000 g for 2 hour. The supernatant was removed, and the viral particles were resuspended in Opti-MEM. The viral stock was titrated and alliquoted into tubules for storage at -80 °C for later use. The produced shRNA-expressing lentiviral particles were designated as Lenti-shDRiP78 and Lenti-shNHERF1, respective.

Table 1 Target shRNA sequences and primers of human DRiP78 and NHERF1

Oligo type	Oligo DNA (5′-3′)
Human DRiP78 target sequence	CCGAGGAACUAUGUCAACUUGGACA
shRNA-U6-DRiP78-1F primer	CACCGCCGAGGAACTATGTCAACTTGGACACGAATGTCCAAGTTGACATAGTTCCTCGG
shRNA-U6-DRiP78-1R primer	AAAACCGAGGAACTATGTCAACTTGGACATTCGTGTCCAAGTTGACATAGTTCCTCGGC
Human NHERF1 target sequence	CAGAAGGAGAACAGUCGUGAA
shRNA-U6-NHERF1-2F primer	CACCGCAGAAGGAGAACAGTCGTGAACGAATTCACGACTGTTCTCCTTCTG
shRNA-U6-NHERF1-2R primer	AAAACAGAAGGAGAACAGTCGTGAATTCGTTCACGACTGTTCTCCTTCTGC

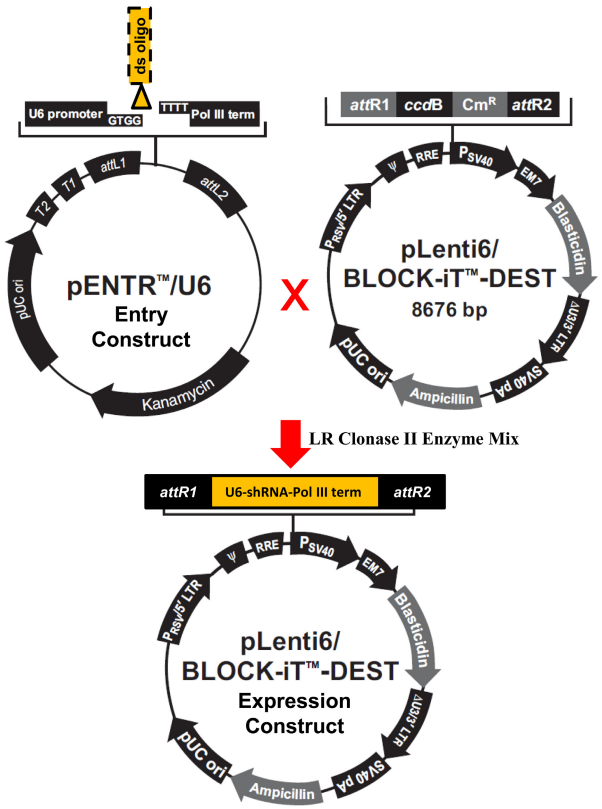

Figure 1 Construction of the shRNA-expressing lentiviral vector

The synthesized complementary oligos (shRNA of target gene) were annealed to produce the double strand oligo (ds oligo, highlighted in yellow). The ds oligo was inserted into the pENTR/U6 vector to generate the pENTR/U6-shRNA construct. The pENTR/U6-shRNA was recombined with the destination vector pLenti6/BLOCK-iT-DEST to finally generate the pLenti6/BLOCK-iT-DEST-shRNA lentiviral expression construct.

Cell culture

HEK293T cells were cultured in DMEM complete medium with 10% FBS, 100 mg/mL Penicillin and 100 U Streptomycin, at 37 °C, 5% CO_2. The GHOST(3)-CXCR4 and GHOST(3)-CCR5 were obtained from Dr. Vineet N. KewalRamani and Dr. Dan R. Littman through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Mörner et al, 1999). The GHOST(3) cells were cultured in DMEM complete medium, supplemented with 500 μg/mL G418 (CalBiochem), 100 μg/mL Hygromycin (CalBiochem) and 1 μg/mL Puromycin (CalBiochem) in a 37 °C, 5% CO_2 incubator.

Blasticidin killing assay

GHOST(3)-CXCR4 and GHOST(3)-CCR5 cells at log state were collected and plated at 3×10^4 cells/well in 24-well plate with 2 mL DMEM complete medium. Cells were allowed to adhere overnight and were at approximately 25% confluence. A series concentration of Blasticidin (0, 2, 4, 6, 8, and 10 μg /mL) was added and cells were incubated at 37 °C, 5% CO_2. The selective media was replenished every 3-4 days, and the percentage of surviving cells was observed. Two weeks later, the viability of cells were observed under microscope to determine the sensible concentration of GHOST(3) cells to Blasticidin.

Lentiviral infection and stable cell selection

GHOST-CXCR4 and GHOST-CCR5 cells were seeded in 1 mL of 12 culture plates, 37 °C, 5% CO_2 overnight, to make sure the cell confluence is around 50%. The medium was changed with DMEM with 5 μg /mL of Polybrene (Santa Cruz) 24 hour later. Lenti-shDRiP78 and Lenti-shNHERF1 and control lentiviral supernatants were thawed in room-temperature water-bath, and then were added into cells; mixed well and incubated at 37 °C, 5% CO_2. Twenty-four hour later, passaged cells at the ratio of 1:5 were cultured overnight. The DMEM medium was changed with sensitive Blasticidin on the next day; and with fresh selection medium on the third day. Two weeks later, cell clones were collected for knockdown efficiency analysis by measuring protein expression levels.

Western Blot

Expression and knockdown efficiency of DRiP78 and NHERF1in GHOST(3) cells was detected by Western Blot. The stable-knockdown GHOST(3)-CXCR4 and GHOST(3)-CCR5 cells were collected and centrifuged, and then the supernatants were dispersed; RIPA lysis buffer (with EDTA-free protease inhibitor cocktail and PhosSTOP) was added, mixed well and set on ice for 30 min, mixed every 5 min; centrifuged at 12 000 g, 4 °C for 10 min. Cell lysates were transferred to a new tube, and protein concentrations were measured by the Bradford Reagent Ready-to-use Protein Measurement Kit (Kangcheng). Equal amount of proteins were separated in 10% SDS-PAGE, and then proteins were transferred to PVDF membrane (Millipore) via Semi-Dry electronic transfer. The membranes were blocked in 5% fat-skim milk for 2 hour at room temperature. The rabbit anti-DRiP78 polyclonal (Sigma-Aldrich) and rabbit anti-NHERF1 monoclonal (Cell Signaling) were incubated with the membrane overnight at 4 °C. The mouse anti-GAPDH monoclonal (Kangcheng) was used as a loading control. On the second day, membranes were washed in 0.05% Tween-20-TBST for 3 times, 5 min each. The HRP-conjugated goat anti-rabbit and anti-mouse second antibodies were then incubated with the PVDF membranes for 1 hour at room temperature. The membranes were washed and developed with the Western Lighting Plus ECL reagent (Perkin-Elmer). ImageJ v1.48 software (National Institutes of Health, USA) was used for densitometric quantitation of western blots.

RESULTS

Construction of lentiviral expression vectors and package of lentiviral particles

The constructed lentiviral expression vectors were sequenced and were aligned with the target gene sequences to verify the direction and shRNA sequences in the constructs. The

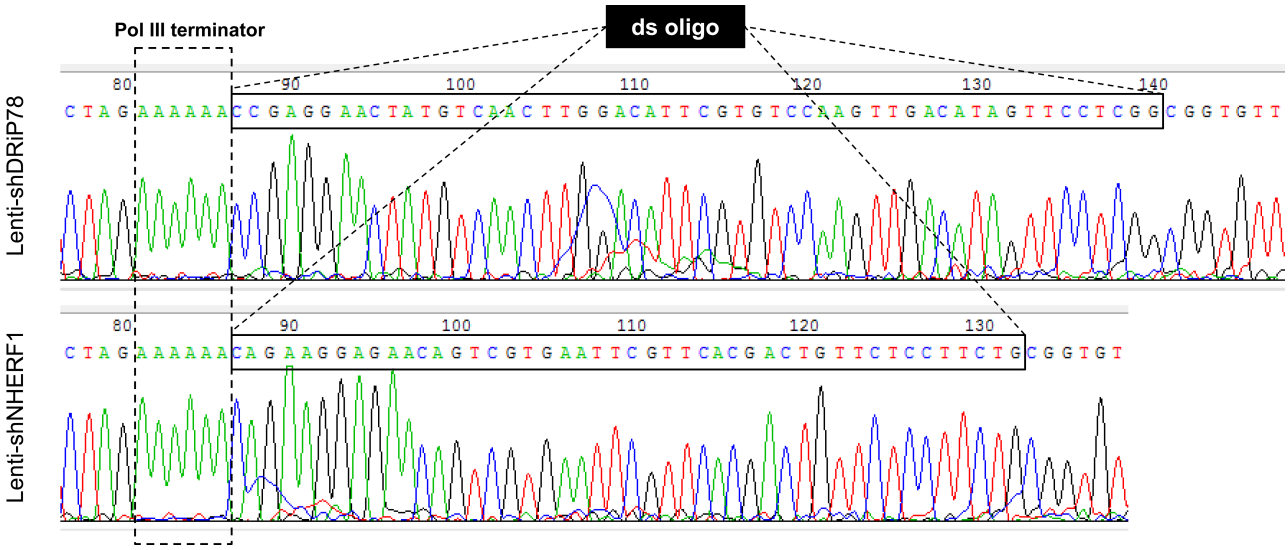

Figure 2 Sequences of inserted shRNA of target genes

The sequences of inserted DRiP78 shRNA (upper panel) and NHERF1 shRNA (lower panel) are shown. The dotted box indicates the Pol III terminator; the boxes indicate the shRNA sequences of DRiP78 (upper) and NHERF1 (lower).

sequencing data showed that the shRNA oligos in the vectors were correct and in the right directions.

Blasticidin sensitivity assay

The sensitive concentrations of HIV-1 susceptible model cell lines GHOST(3)-CXCR4 and GHOST(3)-CCR5 to Blasticidin were determined via killing assay. Cells were plated at approximately 25% confluence and cultured in DMEM complete medium containing 0, 2, 4, 6, 8, and 10 μg/mL of Blasticidin. Selective media were replenished every 3-4 days, and the percentage of survived cells was observed. Two weeks later, the sensitive concentrations of GHOST(3)-CXCR4 and GHOST(3)-CCR5 were determined according to the observations of viable cells in Blasticidin. The microscopic graphs showed that both cell lines were insensitive to the selection media containing 0, 2 and 4 μg/mL of Blasticidin on day 14 post the addition of Blasticidin (Figure 3). The cells were inviable in the selection medium containing 6 μg/mL of Blasticidin. When the selection concentration reached 8 and 10 μg/mL, almost all of the cells were killed.

DRiP78 and NHERF1 expression knockdown in GHOST(3)

The lentivrial particles expressing DRiP78 or NHERF1 shRNA challenged GHOST(3)-CXCR4 and GHOST(3)-CCR5 cells were selected in the selection medium with their sensitive concentrations of Blasticidin. GHOST(3) cells stably knocked down with DRiP78 and NHERF1 were collected and used for protein expression measurement by western blot. In both selected GHOST(3)-CXCR4 and GHOST(3)-CCR5 cell lines, remarkably low expression levels of DRiP78 and NHERF1 were observed (Figure 4). The relative density quantitation of blots showed that in GHOST(3)-CXCR4 cells, the DRiP78 and NHERF1 expressions were decreased by 89.75% and 79.69%,

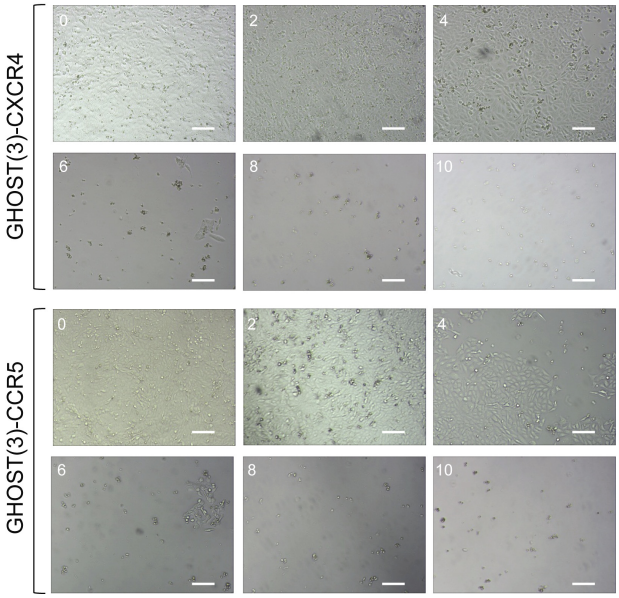

Figure 3 Sensitive concentrations of GHOST(3) cells to Blasticidin

The growth status of GHOST(3)-CXCR4 (upper panel) and GHOST(3)-CCR5 (lower panel) were imaged under microscope on the day 14 post the addition of Blasticidin; The number on the images represents the antibiotic concentration (0, 2, 4, 6, 8, and 10 μg /mL respectively); The magnification of microscope is 10×10; Results were shown of representative data from at least three independent trials; Scale bars=50 μm.

respectively, compared with the control cells (Figure 4),whereas, in the selected GHOST(3)-CCR5 cells, those of DRiP78 and NHERF1 decreased by 78.29% and 74.55%, respectively (Figure 4).

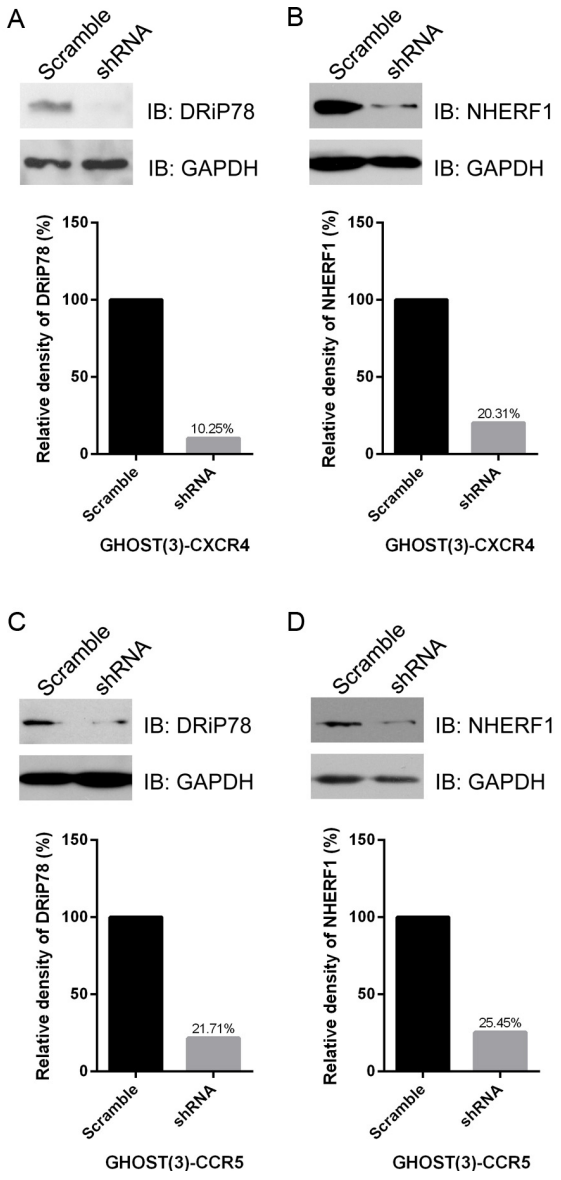

Figure 4 Target protein expressions in stable knocked down GHOST(3) cells

Expression levels of DRiP78 (A) and NHERF1(B) in GHOST(3)-CXCR4 cells and those of DRiP78 (C) and NHERF1 (D) in GHOST(3)-CCR5 cells were detected by western blot; The Scramble and shRNA indicate the control GFP shRNA-transduced cells and target genes (DRiP78 or NHERF1), shRNA-transduced GHOST(3) cells, respectively; The housekeeping GAPDH was used as a loading control; The relative densitometric ratios of each protein to β-tubulin were shown below each panel; Representative data from 3-4 independent trials were shown.

DISCUSSION

Susceptibility of HIV-1 infection, both *in vitro* and *in vivo*, requires interactions between the envelope glycoprotein gp120 and the primary receptor CD4 (Dalgleish et al, 1984; Klatzmann et al, 1984), as well as the co-receptors, either CCR5 or CXCR4, the members of the chemokine receptor family. CCR5-dependent viruses are predominantly responsible for the early stages of infection, such as the inter-individual transmission and the the viral pandemics sustaining. However, the CXCR4-dependent viruses, as well as the dual tropic R5X4, emerge in individuals only at the late, immunologically suppressed stages of diseases (Tsibris & Kuritzkes, 2007). These epidemiological observations imply that the co-receptor-mediated signaling affects the replication efficiency of the virus to its target cells. The identifications of HIV-1 co-receptor-interacting molecules are important in understanding the viral entry and post-entry viral replication proceedings. Our previous studies indicate that NHERF1 interacts with CCR5 via its PDZ2 domain (Hammad et al, 2010; Kuang et al, 2012b). DRiP78 can bind with both CXCR4 and CCR5 (Kuang et al, 2012a).

GHOST(3) cells are derived from human osteo sarcoma (HOS) cells that are stably transduced with MV7neo-T4 (CD4) retroviral vector (Mörner et al, 1999). These cells also contain the gene of green fluorescent protein (GFP) driven by the HIV-2_{ROD} LTR. GHOST(3) cells stably express CD4, as well as CXCR4 and / or CCR5, which are required for infection, through antibiotic resistant selections. The high levels of antibiotics ensure the stability and expressions of both CD4 and its co-receptors. Viral entry activates Tat and the subsequent transcription drives the GFP expression after infection. The ease to use and quick evaluation are the main advantages of the GHOST(3) cell assay. These cells can be used to titer virues, to determine phenotypic properties, and to evaluate drug sensitivities and antibody neutralizations. In this present study, we used the widely-applied BLOCK-iT Lentiviral RNAi Expression system to silence DRiP78 and NHERF1. Blasticidin-selective GHOST(3) cells showed optimal selections at the concentration of 8 μg/mL. The stable GHOST(3) cells selected in Blasticidin medium were morphologically normal compared with cells without transduction (data not shown), suggesting that the expression of shRNA was not detrimental to the GHOST(3) cells. The results of western blot showed that the shRNA sequences used in this work were not off-targeted, and the efficiency in silencing DRiP78 and NHERF1 genes was comparable with the siRNA transfection assay applied in previous studies (Hammad et al, 2010; Kuang et al, 2012a, b; Yi et al, 2011). These findings indicate that the lentiviral RNAi expression system carrying an effective shRNA is an optimal method to silence a target gene, and the shRNA sequences for human DRiP78 and NHERF1 used in this present study can be applied in future works.

In conclusion, here, we have for the first time successfully established the HIV-1 susceptible model GHOST(3) cells expressing receptor CD4 and its co-receptor CXCR4 or CCR5 with stable DRiP78 or NHERF1 knockdown. The establishment of this stable cell line is critical in developing novel anti-viral drugs in the future.

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